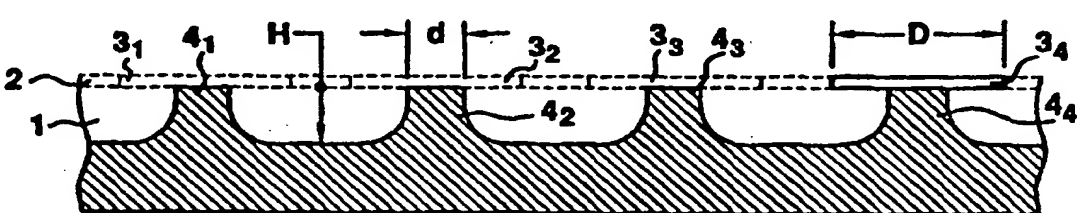


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(54) Title: PROCESS FOR MANUFACTURING OF A SUPPORT PLATE FOR A TWO-DIMENSIONAL NETWORK OF MICROWELLS, PARTICULARLY FOR BIOLOGICAL TESTS OR CULTURES		
		
(57) Abstract The application shows a support plate for microwells for biological tests or cultures, with a very dense network of microwells, and method of making it.		

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PROCESS FOR MANUFACTURING OF A SUPPORT PLATE FOR A TWO
DIMENSIONAL NETWORK OF MICROWELLS, PARTICULARLY FOR
BIOLOGICAL TESTS OR CULTURES

5 The present invention relates to process for
manufacturing of a support plate for a two dimensional
network of microwells, more particularly intended for the
execution of biological tests or cultures. The present
invention also relates to the microwell plate obtained by
10 implementation of this process.

 In order to execute biological tests of cultures, one
currently uses plates molded out of thermoplastic
materials such as a polycarbonate or polystyrene, these
plates having 96 cavities or wells suitable for receiving,
15 for example, cells to be grown and their nutritive medium.
Typically, the plate is rectangular, with an approximately
80 x 125 mm format, and the wells have a diameter of
approximately 8 mm. These dimensions provide about 8 mm on
average. These dimensions are standardized so as to allow
20 for automatic handling of the plates in apparatuses such
as a liquid handling robot, a spectrophotometer, or a
microscope for observation.

 When such plates are used to perform genetic
operations such as grafting of a "probe" on a segment of a
25 DNA molecule, for example, the large volume of the well in
comparison with the dimensions of a cell containing such a
molecule, because of the relative concentration, lowers
the probability of interaction of the probe and the
molecule. Furthermore, the small number (96) of wells

limits the number of tests or cultures which can be executed on the same plate.

The filling of the wells is currently done using pipette assemblies with a high cost of production and requiring specialized personnel for their handling. Each well receives a large number of cells. The grafting of a probe on a DNA molecule of one of them is then randomly carried out, whereas it would be preferable for this grafting to be carried out between a probe and a single cell, which are arranged near one another in a small well in order to increase the probability of execution of the grafting.

Observation of cultures is done through the bottom of the wells, which consists of a transparent membrane or textured or porous wall such as a screen or a fabric. Such means of forming the bottoms of the wells can hinder observation in polarized light, which is often desired, particularly in the case of membranes made of plastic material, which are capable of disturbing the polarization of the observation beam.

The plates mentioned above are currently produced by injection molding of a plastic material. It is known that molding of this type often involves the use of additives, such as lubricants (paraffin, for example) in order to improve the flow properties of the plastic material. Such an additive can later exude into the wells, disturbing the cultures. Other additives, such as products for stabilization of the plastic material with regard to ultraviolet radiation, can also exude and poison the cell cultures. On the other hand, numerous plastic materials exhibit fluorescence, which interferes with the observation of the tests.

There is also known a process for production of a plate made of silicon which, by a conventional process of etching used in the manufacturing of integrated circuits,

is hollowed with a two-dimensional network of wells of very small diameter or "microwells," the density of these microwells per unit of surface area being much greater than that of the wells of the 80 x 125 mm plates described above. The microwells can be filled by wetting of an extended surface of the plate and then removal of the moisture from this plate with retreat of the liquid deposited at the bottom of the microwells. The microwells are delimited by sharp edges which work against the removal of moisture from the surface of the plate by blocking the drops of liquid which flow on the surface of the plate during this moisture removal.

Such silicon plates are fragile, and their manufacturing is delicate, long and costly. Furthermore, since silicon is a particularly opaque material, it is not possible to perform optical observations or measurements through the bottom of the microwells.

Other techniques can be envisaged for producing plates hollowed with a dense network of microwells, for example, the injection molding of the thermoplastic material such as a polycarbonate. The fluorescence of this material and the incorporation, conventional in itself, of lubricants and ultraviolet radiation stabilizers makes it unsuitable for the application in consideration here. It is also possible to think of proceeding by laser machining in order to produce microboring of a substrate. But, then one observes burrs and roughness on the substrate, such that the surface condition of the plate obtained is favorable for the trapping of bubbles and does not offer the quality required to allow moisture to be removed from it.

The present invention therefore aims to provide a process for manufacturing of a plate supporting a very dense two-dimensional network of microwells of very small dimensions, on the order of the dimensions of the living

cells which they are supposed to receive, which ensures easy filling of at least certain ones of the microwells with a small number of cells per microwell, with it possible for the number of wells formed on the plate to be very large so as to allow the execution of a large number of microbiological cultures on the same plate.

The present invention also aims to provide such a process allowing one to produce plates of microwells made of a material with a high degree of chemical neutrality on the surface, not containing or exuding any product capable of disturbing the cultures or hindering their observation.

These aims of the invention, as well as others which will appear upon reading of the following description, are reached with a process for manufacturing of a support plate for a two-dimensional network or microwells for microbiological cultures, which is remarkable in that a) a pressing matrix is formed, made of a nondeformable material with a surface from which projects a two-dimensional network of protuberances isolated from one another and having ends corresponding to the bottoms of the microwells to be formed, these protuberances having a height greater than the depth of said microwells, b) a plate made of a thermoformable material, heated above its softening temperature, is pressed against said matrix surface in such a way that only the protuberances come in contact with said plate and are then sunk into it to a depth equal to that of the microwells to be formed in this plate, and less than the height of these protuberances, and c) said plate is cooled in order to obtain a stable network of microwells on it.

As will subsequently be seen, this process allows one to produce, in a not very expensive manner, in an inert and transparent material such as glass, a network of microwells suitable for receiving cells to be grown in close proximity to probes, according to one of the

essential aims of the invention. The network can be very dense and, for example, have 10^4 times more wells as the 80 x 125 mm plate mentioned above over the same surface area, which allows one to multiply the number of cultures. As an illustrative and nonlimiting example, it is thus possible to manufacture a network of microwells approximately 100 μm apart from one another, these wells having a depth of 20 μm and a diameter at the bottom of 40 μm , making it possible to receive a 10 μm cell to be grown, for example. As will also subsequently be seen, the filling of the microwells occurs by a wetting-moisture removal operation which is particularly convenient and effective.

According to another characteristic of the process according to the invention, the pressing matrix is formed by chemical etching of a metallic plate through a mask consisting of a grid of areas centered on the axes of the protuberances to be obtained.

According to an advantageous variant of the process according to the invention, a number of matrixes are formed, they are stacked with insertion of a plate made of thermoformable material between two adjacent matrixes, and the whole stack thus formed is hot pressed in order to obtain a number of plates, each hollowed with a network of microwells.

The process according to the invention allows one to obtain a plate which bears, on at least one of its surfaces, a network of microwells with an axial section in the form of a funnel which is flared starting from the bottom of the microwell, these bottoms being separated by surfaces which are convex at any point and free of any discontinuity of curvature, these surfaces moreover having a finish of optical quality. This geometry and this finish contribute towards concentrating the liquid products or other products deposited on the plate at the bottom.

Other characteristics and advantages of the process

and of the plate according to the invention will appear upon reading of the following description and upon examination of the appended drawing in which:

- Figure 1 is a diagrammatic and enlarged cross section of a part of a pressing matrix used in the process according to the invention, this view also illustrating its process of manufacturing,

- Figure 2 diagrammatically illustrates the process for manufacturing of the network of microwells according to the invention,

Figure 3 diagrammatically illustrates an advantageous variant of the process of manufacturing represented in Figure 2, allowing one to produce several networks simultaneously,

- Figure 4 is an enlarged view in cross section of a part of a plate according to the invention, which has two networks of microwells, each formed on one of its two opposite surfaces,

- Figure 5 is a picture taken with the scanning electron microscope of a part of the surface of the matrix used in the process according to the invention, and

- Figure 6 is a view taken with the scanning mechanical microscope of a part of a plate obtained by implementation of the process according to the invention.

Thus, as indicated above, the process for manufacturing of a support plate for a network of microwells according to the invention calls for a matrix which allows one to form these microwells by pressing of a plate made of thermoformable material heated to above its softening temperature. This material can be a glassy material such as glass, or a transparent plastic material.

In order to produce this matrix, according to a preferred mode of implementation of the invention, one starts with plate 1 made of a stable metallic alloy such as Nicrimphy of IMPHY SA.

A surface of metallic plate 1 is covered with a photosensitive resin which is exposed and strips off, by a conventional technique of photolithography, in such a way as to leave remaining on the surface of the plate a mask with the form of a regular grid of areas such as those referenced $3_1, 3_2, 3_3, \dots$ in Figure 1, with a roughly circular shape, for example.

Plate 1 is then chemically etched through this mask, using an etching solution which does not attack the resin of areas 3_i . In order to etch the Nicrimphy, it is possible to use a solution of iron perchloride with addition of 20 to 30% nitric acid. It is known that the isotropic attack of the metal of plate 1 by the etching solution dissolves the metal under area 3_i over a distance H equal to the desired etching depth. If one then wishes a network of protuberances 4_i with an end diameter d to appear on the surface of plate 1, it is then necessary to expose the photosensitive resin in such a way as to cause the appearance, after stripping, of areas 3_i of resin with diameter D such that:

$$D = d + 2H$$

With the etching of the metal finished, areas 3_i of resin are removed with a suitable solvent, and the etched surface of plate 1 then has the appearance illustrated by the view of Figure 5, taken with the scanning electron microscope. In this view appears a regular network of protuberances 4_i flared starting from their end and rejoining a bottom whose roughness is apparent in the view presented.

Editor's note: With the exceptions of 4_i and 10_i in Figures 5 and 6 respectively, no subscript "i" occurs in any of the figures. Therefore, subscript i should in many cases probably be read as subscript 1.

Plate 1 thus etched constitutes pressing matrix 5 which can be used in the process for manufacturing of plates of microwells according to the invention, illustrated in Figure 2. Typically, the protuberances are approximately 100 to 200 μm apart, are 20 to 50 μm high, and have an end diameter between 20 and 50 μm .

For this purpose, as represented diagrammatically in this figure, one applies the etched surface of matrix 5 against plate 6 made of a thermoformable material heated to a sufficient temperature for it to have suitable plasticity or viscosity to be pressed. This material can be a plastic material, or more preferably, glass such as that referenced 7059 in the catalogues of the company Corning Incorporated. In the latter case, matrix 5 and glass plate 6 are placed in a furnace and gradually brought to a temperature of 740°C , so that the glass has a viscosity of approximately 10^{10} P. At this viscosity, suitable pressing of the matrix 5-plate 6 assembly using pressure plates 7,8 for approximately 1 min, causes protuberances 4₁ of the matrix to penetrate into glass plate 6 to a depth which can be controlled so that glass surface elements 9₁, 9₂, 9₃, ... separating the wells 10₁, 10₂, 10₃, ... hollowed by protuberances 4₁ in plate 6 in the plastic state, keep an "optical" finish because of the absence of any contact with surface elements 11₁, 11₂, ... of the relatively rough bottom of the matrix.

After cooling of plate 6 to room temperature, this plate bears a regular two-dimensional network of microwells 10₁ such as those which appear in Figure 6, which is a view of the surface of the plate taken with the scanning mechanical microscope.

In this view, it appears that the wells have a funnel shape, regularly flared from the bottom of the wells towards their openings on the surface of the plate, the bottoms being separated by surfaces which are convex at

any point, which are free of any discontinuity of curvature, and have a finish of optical quality. It should be observed that these surfaces converge regularly towards the bottoms of the adjacent microwells.

5 Thanks to these shape and surface properties, it is easy to fill the microwells with cells to be grown, carried, for example, in a nutritive medium. A drop of a medium thus loaded with cells deposited on the surface of the plate in such a way as to "wet" the plate, the medium
10 and the cells are "sucked" towards the bottoms of the wells by a process of "moisture removal" of the curved and smooth surfaces bordering the wells. The excess can be evacuated by controlled evaporation. The plate is then protected by a thin sheet of glass.

15 The process according to the invention allows one to produce plates with a very high density of microwells, for example 10^4 wells per cm^2 . It is possible then, by the process of wetting-moisture removal described above, to provide a very large number of microwells with cells to be
20 grown and their nutritive medium, the "probes" having been previously deposited according to a predetermined cartography in the wells. Typically, as an example, the microwells can have a depth of 15 to 30 μm , the diameter of the bottom of the wells being in the range of 20-50 μm ,
25 and the distance between the wells being approximately 100 to 200 μm . It is understood that a cell, typically approximately 10 μm , is then found at the bottom of a well very close to the probes introduced beforehand into this well, which very advantageously increases the probability
30 of the desired grafting.

 Texturing of the bottom of the microwells facilitates suction and retaining of the cells and drops of liquid deposited on the surface of the plate of microwells. This texturing can be obtained, according to the invention, by
35 rolling, brushing, polishing, glazing, shot-peening, ion

bombardment, for example, of the surface of the metal plate used to produce the pressing matrix, before deposition of the photosensitive resin intended for the formation of the etching mask. Thus, the end surfaces of protuberances 4, formed later by chemical etching, have a texture, the copy of which is printed on the bottom of the microwells during pressing of plate 6 according to the invention.

The end surfaces of the protuberances could also be hollowed, by "Eximer" laser etching, for example, with identification marks such as numbers or bar codes, for example, marks whose copy is transferred to the bottoms of the microwells at the time of pressing of the plate, as described above. The marking of the various cultures carried by the plate is facilitated by these means.

The plate of microwells according to the invention can carry other marks allowing for automatic identification of the plate in apparatuses for manipulation or treatment. These marks can also be formed during pressing by copying corresponding marks formed on the matrix.

It should be observed that the present invention allows one to make it so that the bottoms of the wells of the same plate are all coplanar, which facilitates focusing of a microscope for observation of these cultures.

In this regard, the network of microwells can have lines or columns of microwells with bottoms whose shape, for example, oval, differs from that of the others (for example, circular). These lines and columns, regularly distributed, provides a meshwork for the network which can be observed with the microscope, which facilitates exploration of the network and identification of the cultures made in the microwells.

Techniques of pressing other than the static pressing

illustrated in Figure 2 could be used to manufacture the plate of microwells according to the invention, for example, rolling, blowing, or suction of a material in the plastic state, against a matrix according to the invention.

A variant of the process according to the invention is illustrated in Figure 3. According to this variant, several matrixes $5_1, 5_2, 5_3, \dots$ which are stacked with insertion of plate $6_1, 6_2, 6_3, \dots$ made of thermoplastic material between two adjacent matrixes. The whole is heated and then pressed as described above in connection with Figure 2. This variant has two advantages: on one hand, it increases the productivity of the process, and, on the other hand, it improves the regularity of the pressing operations done on the various plates 6_i by the "mattress" effect resulting from the stacking of the plates, an effect which is favorable for making the pressure applied on the plates uniform.

A variant of the plate of microwells according to the invention is represented in Figure 4, by a cross section of this plate according to a plane perpendicular to its surface. The section represented shows that each of the two surfaces of plate $6'$ has a network of microwells $10_1, 10_2, \dots$ and $11_1, 11_2, \dots$ respectively. The network of microwells 10_i roughly corresponds to that of the plate of Figure 2. Microwells $11_i, \dots$ formed on the other surface of the plate are each centered on the axis of a microwell 10_i . Their size is determined so as to receive a means of treatment (by heating, for example) or of observation of the culture performed in the corresponding microwell 10_i . This means can take the form of one or more optical fibers 12 , for example, in the case of automatic analyses by fluorescence or colorimetry.

The two networks of microwells can be used to execute cultures. Flat glass plates applied against the networks

of microwells can protect the cultures which are developed in them.

It now appears that the present invention indeed allows one to reach the aims and to obtain the advantages which were chosen. Thus, thanks to the great density of microwells formed in a plate and thanks to the geometry of these microwells, bordered by convex surfaces converging towards the bottoms of the adjacent wells, it is possible to easily fill a large number of wells with liquid media and cells necessary for a microbiological culture of these cells, for purposes of genetic engineering, for example.

The material preferably used for forming the plate, namely glass, contrary to the majority of the plastic materials, allows for observations of cultures in polarized light as well as in natural light, undisturbed by interfering fluorescence.

The high degree of chemical inertness of glass allows for the use of acetone, which is necessary for fixing the cells, or for the use of stains such as, for example, hematoxylin, which is necessary for observation of the contents of the nuclei, and at the same time, it prevents contamination by products coming from the material of the plate.

The excellent dimensional stability of glass contributes several other advantages: great precision (on the order of a micrometer) of identification of the wells on the plates, as well as of positioning of the plates in machines for the operation of treatments, easy focusing of an observation microscope on the flat and coplanar bottoms of the wells.

Using a velvet pad, it is possible to transfer simultaneously a part of the contents of each well in order to transfer them to another plate, which allows one to copy these cultures.

The flared shape of the microwells allows possible

microbubbles of air to escape from the wells rather than to remain caught there, disturbing the cultures and the observations of which they are the object.

5 The very small size of the microwells allows one to execute, in each of them, monocellular cultures or cultures of a very small number of cells, allowing one to obtain clones whose paternity is certain. This small size of the wells increases the relative concentrations and promotes the probability of interaction of the probes (DNA
10 fragments) with the molecules of DNA on which grafting is supposed to be done.

 Polymerase chain amplification, commonly called PCR, which requires thermal cycling, would not be possible "in situ" with the plates made of thermoplastic material (for
15 example, polystyrene) currently used for cell cultures, but it becomes possible with a glass plate because of the very good resistance of this material to such cyclings.

 Finally, particularly advantageously, the invention allows one to manufacture support plates for very dense
20 networks of microwells by a pressing process, allowing for manufacturing of said plates at industrial speeds and at particularly low actual manufacturing costs.

 Of course, the invention is not limited to the embodiment described or represented, which was only given
25 as an example. Thus, the plate according to the invention could also be produced with various plastic materials, insofar as the problems of contamination, or difficulties of observation of the cultures mentioned above do not arise under the conditions of use of such a plate. A
30 ductile metal capable of plastic deformation could also be used, if observations made through the bottoms of the microwells are not foreseen. Furthermore, the invention applies not only to the execution of biological cultures but also to the execution of any test or treatment of
35 microsamples of any materials.

Claims

1. A process for manufacturing of a support plate for a two dimensional network of microwells for biological tests or cultures, characterized in that:

a) pressing matrix (5; 5_i) is formed, made of a nondeformable material with a surface from which projects a two-dimensional network of protuberances (4_i) isolated from one another and having ends corresponding to the bottoms of the microwells to be formed, these protuberances having a height greater than the depth of said microwells (10_i),

b) plate (6; 6_i) made of a thermoformable material, heated above its softening temperature, is pressed against said matrix surface (5; 5_i) in such a way that only protuberances (4_i) come in contact with said plate and are then sunken into it to a depth equal to that of microwells (10_i) to be formed in this plate, and less than the height of these protuberances, and

c) said plate (6; 6_i) is cooled in order to obtain a stable network of microwells on it.

2. A process according to Claim 1, characterized in that matrix (5; 5_i) is formed by chemical etching of a metallic plate, through a mask consisting of a grid of areas (3_i) centered on the axes of the protuberances to be obtained.

3. A process according to either of Claims 1 and 2, characterized in that a plate made of glass is pressed against said matrix (5; 5_i).

4. A process according to any one of Claims 1 to 3, characterized in that, before forming protuberances (4_i) on the matrix, the surface from which these protuberances are

formed is textured by any one of the processes of the group formed by: rolling, glazing, polishing, shot-peening, ion bombardment.

5 5. A process according to any one of Claims 1 to 4, characterized in that raised identification marks are formed on the ends of protuberances (10_i) of matrix (5; 5_i).

10 6. A process according to any one of Claims 1 to 5, characterized in that a number of matrixes (5_i) are formed, they are stacked with insertion of plate (6_i) made of thermoformable material between two adjacent matrixes, and the whole stack thus formed is hot pressed in order to
15 obtain a number of plates, each hollowed with a network of microwells (10_i).

 7. A process according to any one of Claims 1 to 6, characterized in that the two surfaces of a plate made of
20 thermoformable material are simultaneously pressed against two matrixes of protuberances, these matrixes being arranged in such a way that each protuberance (10_i) of one is coaxial with a protuberance (11_i) of the other.

25 8. A process according to any one of the preceding claims, characterized in that matrix (5; 5_i) is pressed against plate (6; 6_i) by one of the processes of the group formed by: static pressing, rolling, blowing, suction.

30 9. A process according to any one of Claims 1 to 8, characterized in that a matrix is used whose protuberances are approximately 100 to 200 µm apart, 20 to 50 µm high, and of which the end diameter is between 20 and 50 µm.

35 10. A support plate for a two-dimensional network of

microwells obtained by the process of manufacturing according to Claim 1, characterized in that on at least one of its surfaces, it has a network of microwells (10_i), with an axial section in the form of a funnel flared starting from the bottom of the microwell, these bottoms being separated by surfaces (9_i) which are convex at any point and free of any discontinuity of curvature, these surfaces moreover having a finish of optical quality.

10 11. A plate according to Claim 10, characterized in that the bottoms of microwells (10_i) are textured.

15 12. A plate according to Claim 10, characterized in that the bottoms of microwells (10_i) have impressions of raised identification marks.

20 13. A plate according to Claim 10, characterized in that the network of microwells (10_i) has lines or columns of microwells with bottoms whose shape differs from that of the others microwells, these lines and columns providing a meshwork for the network which can be identified optically.

25 14. A plate according to any one of Claims 10 to 13, characterized in that it has two networks of microwells (10_i; 11_i) each formed on one of its two opposite surfaces, each of microwells (10_i) of one of the networks being coaxial with one of microwells (11_i) of the other network, and each of the microwells of one of the networks being
30 configured so as to receive a means of treatment or observation (12) of a culture executed in coaxial microwell (10_i) of the other network.

35 15. A plate according to Claim 10, characterized in that said means of observation (12) consists of an end of

an optical fiber.

16. A plate according to any one of Claims 10 to 15,
characterized in that the axes of wells (10_i) are
5 approximately 100 to 200 μm apart, the depth of the wells
being between approximately 15 and 30 μm , while the
diameter of the bottom of the well is between
approximately 20 and 50 μm .

10 17. A plate according to any one of Claims 10 to 16,
characterized in that it consists of a material of the
group formed by: a glassy material, a glass, a plastic
material, a plastically deformed ductile material.

15 18. Pressing matrix (5; 5_i) obtained by the process
according to Claim 2.

FIG.1

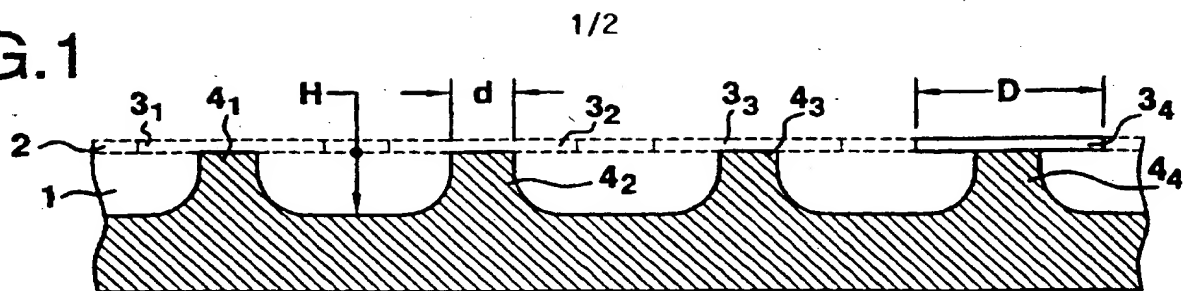


FIG.2

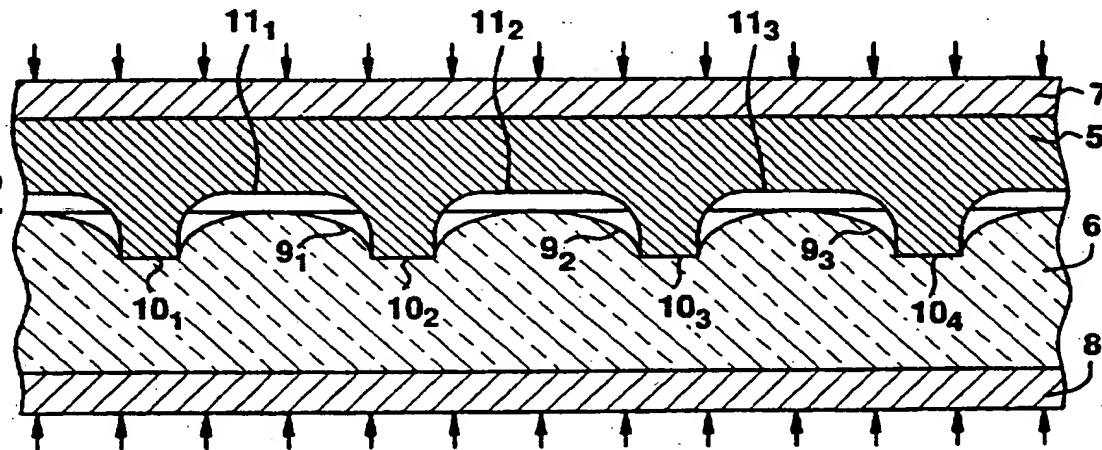


FIG.3

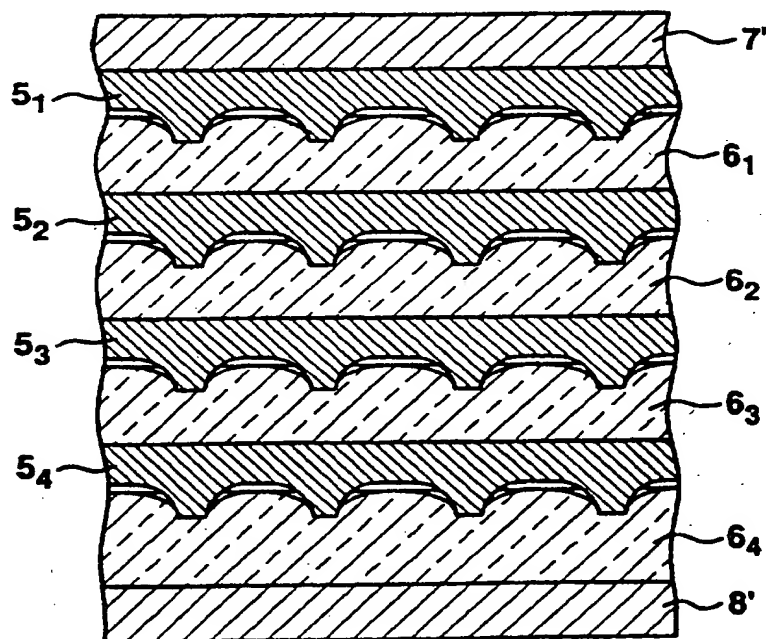
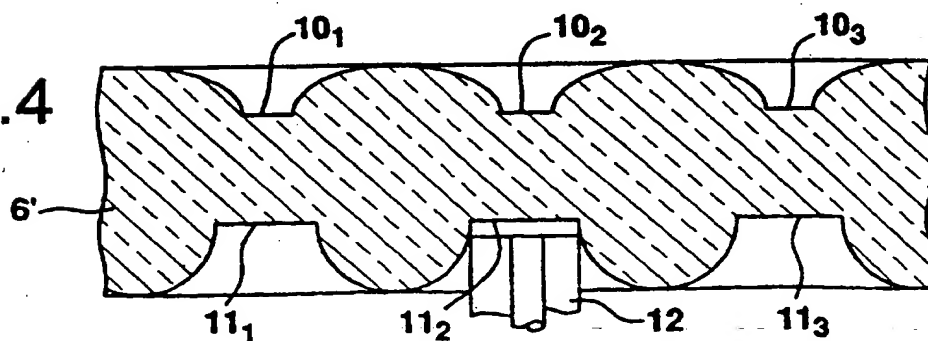


FIG.4

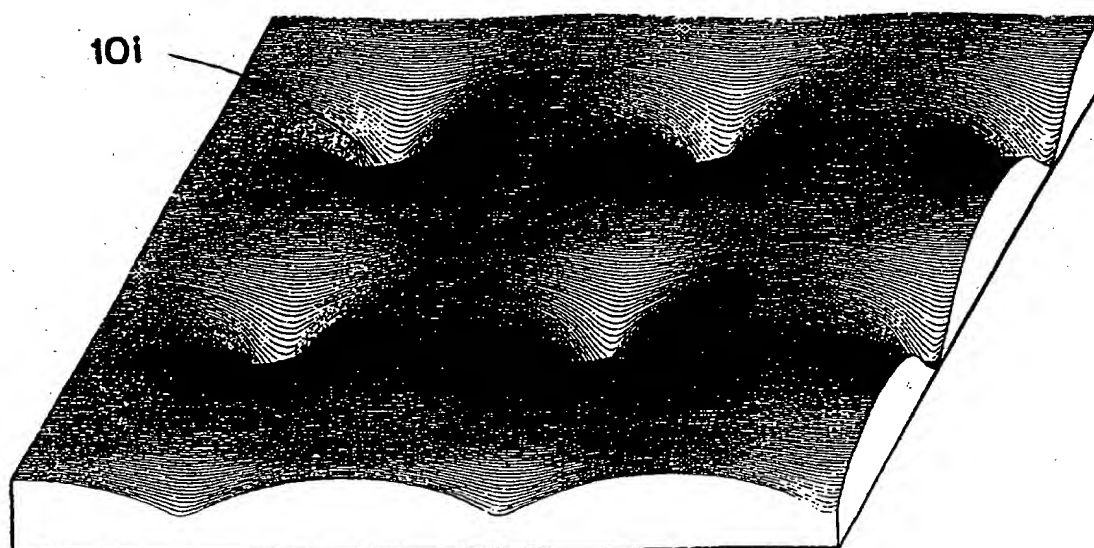


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FIG. 5



FIG. 6



INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 96/18609

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C03B23/00 C03B23/02 C03B11/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C03B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 580 112 (MATSUSHITA ELECTRIC INDUSTRIAL CO., LTD.) 26 January 1994 see the whole document ---	1,3,8, 10,18
X	US,A,3 961 929 (STOCKDALE) 8 June 1976 see the whole document ---	1,3,8, 10,18
X	WO,A,89 05507 (PILKINGTON PLC) 15 June 1989 see the whole document ---	1
A	FR,A,2 184 579 (CORNING GLASS WORKS) 28 December 1973 see the whole document ---	1,10,18

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Date of the actual completion of the international search

24 January 1997

Date of mailing of the international search report

7.02.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
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INTERNATIONAL SEARCH REPORT

Internat. Application No
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